PERGAMON

Phytochemistry 62 (2003) 213–218

PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

Inhibition of LPS-induced NO production by the oleogum resin of *Commiphora wightii* and its constituents

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Received 14 February 2002; received in revised form 13 August 2002

Abstract

Three new (**1**–**3**) and five known compounds (**4**–**8**) were isolated from the oleogum resin of *Commiphora wightii* (Arnott.) Bhanol. Their structures were elucidated by spectroscopic and chemical methods. The MeOH extract and the EtOAc-sol. fraction were found to demonstrate significant inhibition of NO formation in lipopolysaccharide (LPS)-activated murine macrophages J774.1 in vitro (IC_{50} values of 16.4 and 12.8 μg/ml, respectively). When compared with curcumin (IC_{50} value of 12.3 μM), *Z*- and *E*-Guggulsterones (**4** and **5**, respectively) were the most potent inhibitors of NO production (IC_{50} values of 1.1 and 3.3 μM, respectively), followed by myrrhanol A (**7**) and myrrhanone A (**8**) (IC_{50} values of 21.1 and 42.3 μM, respectively). Guggulsterone-M (**1**) and its didehydro derivative (**2**) were weak inhibitors, while guggulsterols I (**6**) and Y (**3**) were inactive ($IC_{50} > 500$ μM).

Keywords: *Commiphora wightii*; Burseraceae; Oleogum resin; Steroids; LPS-induced nitric oxide; Murine macrophages J774.1

1. Introduction

Nitric oxide (NO) is an important mediator released by murine macrophages and other cells after activation. In response to cytokines and other inflammatory stimuli such as bacterial lipopolysaccharides (LPS), NO is synthesized from L-argenine by the inducible enzyme, nitric oxide synthase (iNOS) (Moncada, 1999; Salvemini et al., 1996; Stuehr et al., 1987). There is growing evidence that overproduction of NO is associated with oxidative stress and with the pathophysiology of various diseases such as rheumatoid arthritis, diabetes, cardiovascular diseases, and chronic inflammation (Moncada et al., 1991). In the search for biologically active compounds from crude drugs that are employed in traditional medicine, the MeOH extract of the oleogum resin of *Commiphora wightii* was found to demonstrate potent inhibitory activity of NO production in LPS-activated murine macrophages with IC_{50} value of 16.4 μg/ml) (Table 1).

C. wightii (Arnott.) Bhanol. [syn. = *C. mukul* (Hook, ex Stocks) Engl.] (Fam. Burseraceae) (Sarin, 1996) is endemic to the Indian peninsula and grows wild in the arid and semi-arid regions of Rajasthan, as well as Sind in Pakistan. The oleogum resin of *C. wightii* (known as guggul) was reported to be efficacious in the treatment of rheumatoid arthritis, obesity and allied disorders (Nadkarni, 1954). This exudate possesses a variety of pharmacological activities; anti-inflammatory, antirheumatic (Dash, 1974), and hypolipidemic (Satyavati, 1991). Most of these activities are due to the presence, among the secondary metabolites, of a series of steroids, named guggulsterols (such as **4**–**6**), (Bajaj and Dev, 1982; Patil et al., 1972; Kumar and Dev, 1987). Guggulipid, the EtOAc-soluble fraction of guggul, was found to offer considerable benefit for preventing and treating atherosclerotic vascular disease. *Z*- and *E*-Guggulsterones (**4** and **5**, respectively) showed significant inhibition of platelet aggregation (Mester et al., 1979), total serum lipid and total cholesterol (Satyavati, 1991). Other biologically active compounds were recently isolated from guggul (Kimura et al., 2001; Zhu et al., 2001). In order to validate the traditional use of guggul as antiinflammatory remedy and to clarify its pharmacological effects, the MeOH extract of guggul was investigated with the specific objective of identifying the constituents responsible for the inhibitory effect of guggul

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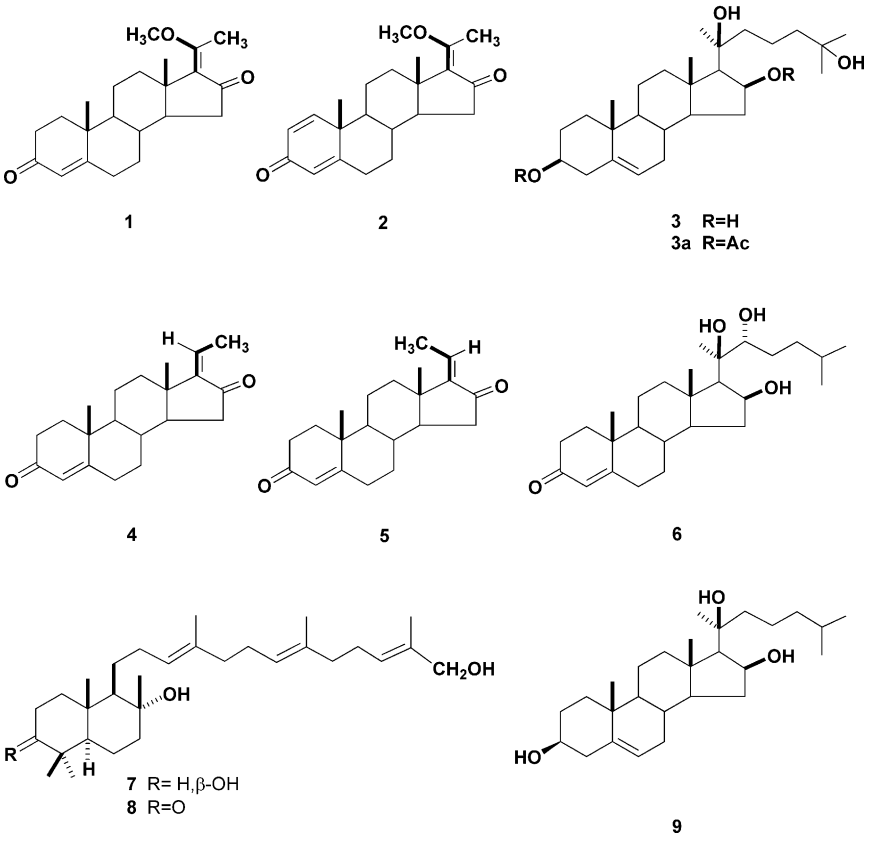
PII: S0031-9422(02)00388-6



on LPS-induced NO production in murine macrophage J774.1 cells. Subsequent fractionation of the MeOH extract has led to the isolation and characterization of eight compounds (including three new) from the EtOAc-soluble fraction. This paper reports the isolation and structure elucidation of these compounds, as well as their inhibitory effects of LPS-induced NO formation.

2. Results and discussion

Through a bioactivity-guided fractionation of the MeOH extract of the oleogum resin of *C. wightii*, the NO inhibitory activity was enriched in the EtOAc-sol. fraction (IC_{50} value of 12.8 μg/ml) from which eight compounds were isolated. Of them, five compounds (**4**–**8**) were previously isolated from the title plant and their structures were identified as *Z*-guggulsterone (**4**), *E*-guggulsterone (**5**), guggulsterol-I (**6**), myrrhanol A (**7**) and myrrhanone A (**8**) by direct comparison of their spectral data with literature values (Patil et al., 1972; Bajaj and Dev, 1982; Benn and Dodson, 1964; Kimura et al., 2001). Compounds **1**–**3** were new and their structures were determined as follows.

Table 1
Inhibitory activity of **1**–**8** on LPS-induced NO production in murine macrophage J774.1 cells

Sample	IC_{50} (μM)	Sample	IC_{50} (μM)
MeOH Ext.	16.4 (μg/ml)	**4**	1.1
EtOAc-sol. Fr.	12.8 (μg/ml)	**5**	3.3
		6	> 500
1	265.0	**7**	21.1
2	78.5	**8**	42.3
3	> 500	Curcumin	12.3

Guggulsterone M (**1**) was obtained as colorless needle crystals (mp 206–208 °C, MeCN) and gave a molecular ion peak at *m/z* 342 $[M]^+$ in the EIMS consistent with the molecular formula $C_{22}H_{30}O_3$, which was further confirmed by high resolution EIMS. The IR spectrum of **1** showed absorptions at 1668 (α,β-unsaturated ketone), 1635 and 1590 (C=C) cm^{-1}. Most of the NMR spectral data of **1** were typical of C_{21}-ketosteroids with shift values resembled those of **4** and **5**. Signals for five quaternary carbons at δ_c 199.5, 195.5, 170.6, 170.7 and 125.9, and one methine carbon at δ_c 124.0 indicated the presence of α,β-unsaturated ketone functions. The ^{1}H-NMR spectrum of **1** analyzed by ^{1}H–^{1}H COSY and HMQC showed three singlets at δ 0.98, 1.21, 2.28 assigned for two tertiary methyls, and an olefinic methyl group, respectively. Two singlets at δ 3.82 (3H) and 5.74 (1H) pointed to the presence of a methoxyl group and an olefinic methine in **1** (signals for two olefinic methines were observed in the spectra of **4** and **5**). This information suggested that one of the olefinic methins, in **4** and **5**, was replaced by a methoxyl group, in **1**. This

was supported by EIMS, which displayed $[M]^+$ at 14 mass units more than those of **4** and **5**. Placement of the methoxyl group at C-20 was confirmed by long-range correlation observed in the HMBC spectrum between C-20 (δ_c 170.7) and ^1H signals at δ 2.28 (3H, *s*, CH_3-21) and 3.82 (3H, *s*, $-OCH_3$). In the NOE difference experiment of **1**, irradiation of the proton signal at δ 3.82 ($-OCH_3$) showed significant enhancement of the proton signal at δ 2.42 (H_a-12) and 0.98 (H_3-18) (Fig. 1), while irradiation of $C\underline{H}_3$-21 only showed enhancement of $-OC\underline{H}_3$ protons. This finding established the geometry about 17(20) double bond in **1** to be *E*. On the bases of the above findings, the structure of guggulsterone-M (**1**) was established as 20-methoxy-4,17(20)*E*-pregnadiene-3,16-dione.

Dehydroguggulsterone M (**2**) was obtained as amorphous powder, $[\alpha]_D$ +36.5° (*c* 0.76, MeOH). The ^{13}C NMR experiments of **2**, including DEPT, sorted 22 signals into four methyl, five methylene, six methines and seven quaternary carbons, with shift values almost similar to those of **1**, **4** and **5**. The IR spectrum established the presence of α,β-unsaturated ketone functions. The NMR spectral data of **2** were in part similar to those of **1**. However, C-1 and C-2 were displayed downfield at δ_c 155.6 (δ_H 6.94) and 127.5 (δ_H 6.13), suggesting the presence of an additional double bond. HMQC and HMBC spectra further confirmed the above inference and revealed that **2** (EIMS *m*/*z* 340 $[M]^+$) was the 1,2-didehydro derivative of **1**. From the NOE difference experiment, the geometry about 17(20) double bond in **2** was established to be *E*, as in **1** [both H_a-12 (δ_H 2.35) and H_3-18 (δ_H 0.88) were enhanced on irradiation of $-OC\underline{H}_3$ protons at (δ_H 3.69)]. On the bases of the above findings, the structure of **2** was established as 20-methoxy-1,4,17(20)*E*-pregnatriene-3,16-dione.

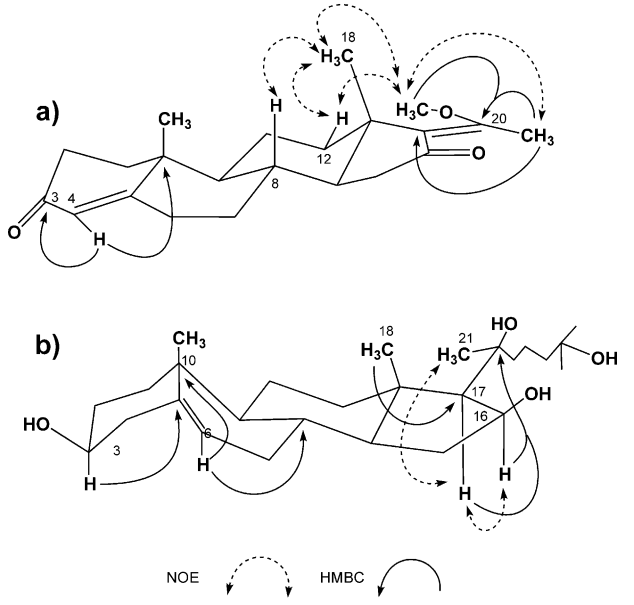


Fig. 1. Significant NOEs and long-range correlations observed in NOE-difference (or NOESY) and HMBC experiments of: (a) guggulsterone-M (**1**); (b) guggulsterol-Y (**3**).

Guggulsterol Y (**3**) gave a molecular ion peak at *m*/*z* 434.3410 in the HR-EIMS corresponding to the molecular formula $C_{27}H_{46}O_4$ (requires 434.3424). The IR spectrum showed absorptions for hydroxyl (3334 cm^{-1}) and olefinic (1666 cm^{-1}) functions. The NMR spectra of **3** analyzed by ^1H–^1H COSY and HMQC showed features characteristic for a C_{27}-steroids similar to guggulsterol-II (**9**) (fragment ions at *m*/*z* 279 [M-side chain]$^+$ and 252 were observed in the EIMS spectrum of **3**) (Patil et al., 1972). However, the presence of 5 singlets at δ 0.98, 1.00, 1.14, 1.24 and 1.36 for 5 angular methyls, and the presence of ^{13}C-signals at δ 76.0 and 77.2, assignable to two oxygen-bearing quaternary carbons, suggested different side chain as depicted in **3**. Besides, signals at δ 3.40, 4.56 and 5.33 were assigned for H-3 (C-3 at δ 72.4, *d*), H-16 (C-16 at δ 70.3, *d*), and H-6 (C-6 at δ 120.2, *d*), respectively. On acetylation, **3** gave a diacetyl derivative [**3a**, *m*/*z* 518 $[M]^+$ and 398 $[M-2AcOH]^+$; IR ν_{max} 3330 (–OH), 1746 and 1740 (ester C=O) cm^{-1}]. ^1H NMR spectrum of **3a** showed two singlets at δ 1.96 (3H) and 2.02 (3H) for two acetyl methyls, and signals for H-3 and H-16 were displayed downfield at δ 4.56 and 5.25 ppm, respectively. This finding indicated that **3** is a tetraol having two tertiary hydroxyl groups. HMBC correlation between the^{13}C signal at δ 76.0 (C-25) and both of H_3-26 and H_3-27, and between C-20 (δ 77.2) and both of H-17 (δ 1.23, 1H, *d*, *J* = 4.4 Hz) and H_3-21 (1.36, 3H, *s*), supported the side chain in **3**. Similarly, correlation between C-16 (δ 70.3) and H-14 (δ 1.15, *m*) and H-17 supported the substitution in ring D to be as shown in **3**. The muliplicity of H-3 is indicative of a β-configuration of C_3-OH, and NOESY correlations between H-17 and both of H-16 and H_3-21 (Fig. 1) suggested the β-orientation of C_{16}-OH, and established the stereochemistry at C-20 as *S*. Accordingly, the structure of **3** was determined to be 20(*S*)-cholest-5-en-3β,16β,20,25-tetrol.

The MeOH extract and the EtOAc-soluble fraction of guggul showed significant inhibition of NO formation in LPS-induced macrophages with IC_{50} values of 16.4 and 12.8 μg/ml, respectively. When compared with curcumin (IC_{50} value of 12.3 μM) used as positive control in the present experiment, *Z*-guggulsterone (**4**) and its isomer (**5**) were the most potent inhibitors of NO production IC_{50} values of 1.1 and 3.3 μM, respectively. Next in potency was myrrhanol A (IC_{50} = 21.1 μM), which was 2 times more potent than myrrhanone A (IC_{50} = 42.3 μM). Dehydroguggulsterone-M (**2**) was weak inhibitor of NO-production (IC_{50} = 78.5 μM) followed by guggulsterone-M (**1**). The C_{27}-steroids **3** and **6** were inactive.

In the present study, eight compounds were isolated from guggul, the oleogum resin of *C. wightii*, as inhibitors of NO formation in LPS-primed murine macrophage

J 774.1 cells. In models of acute inflammation, guggulsterones were reported to be as potent as phenylbutazone and ibuprofen, and were more potent than hydrocortisone in reducing the severity of secondary lesions in models of chronic inflammation (Arora et al., 1971; Arora et al., 1972; Sharma & Sharma, 1977). Also, myrrhanol A (**7**) displayed a potent antiinflammatory effect on exudative pouch fluid, angiogenesis, and granuloma weights in adjuvant-induced air-pouch granuloma of mice (Kimura et al., 2001). Besides inflammation, NO is also implicated in many diseases including cardiovascular and neurodegenerative diseases (such as Alzheimer and Parkinson's diseases) (Glasson et al., 2000).

The finding that the guggulsterones, **4** and **5**, as well as the triterpene **7** were major components in guggulipid (the EtOAc-sol. fraction of the MeOH extract) and that they were potent inhibitors of NO is of special significance. It may explain, at least in part, (1) the mechanism of their pharmacological action and their potential as antiinflammatory agents, and (2) the beneficial effect that guggul have in reducing inflammation as well as neurodegenerative diseases.

3. Experimental

3.1. General experimental procedures

Mp. Uncorr., optical rotations were measured with a Jasco DIP-360 digital polarimeter at 25 °C. UV spectra were recorded on a Shimadzu UV-2200 spectrophotometer, whereas IR spectra were obtained on a Jasco FT/IR-230 infrared spectrometer. CD spectra were measured with a Jasco J 805 spectropolarimeter. ^{1}H and ^{13}C spectra (in $CDCl_3$) were recorded on a Jeol JNM-LA-400 spectrometer using TMS as an internal standard. EIMS (70 eV) and high resolution EIMS were performed with a Jeol JMS-GC-mate mass spectrometer. TLC: silica gel 60 F_{254} and RP-18 plates (Merck) using solvent systems A (benzene–MeOH, 90:10) and B (MeOH–H_2O, 80:20), respectively, and spots were detected under a UV lamp and after spraying with $Ce(SO_4)_2/H_2SO_4$ and heating.

3.2. Materials

The oleogum resin exudate of *C. wightii* (Arnott.)Bhanol. [syn. = *C. mukul* (Hook, ex Stocks) Engl.] was purchased from Khari Baovli, crude drug market, Delhi-6, India during October 2001. The material was in the form of light to dark brown conglomerates of tears and was only slightly sticky to touch and had a faint balsamic odor. The material was kindly identified by Dr. Javed Ahmad, Reader, Dept. of Botany, Jamia Hamdad (Hamdad University), New-Delhi 110062, India. Voucher specimen was deposited at the Department of Pharmacognosy, Faculty of Pharmacy, Cairo University. The murine macrophage cell line, J774.1, was obtained from the Riken Cell Bank, Japan. *Escherichia coli* lipopolysaccharide (LPS, *E. coli* Serotype 055:B5) was obtained from Sigma Chemicals (St. Louis, MO, USA). RPMI 1640 medium was from Santa Ana (CA, USA), phosphate-buffered saline (PBS), and heat-inactivated foetal calf serum (FCS) were purchased from Nissui Pharmaceutical Co. Ltd., Tokyo, Japan.

3.3. Isolation procedure

The oleogum resin (250 g) was triturated with MeOH (1 l × 4) and the combined MeOH extracts were evaporated in vacuo to give 56 g of a yellowish brown gum. The MeOH extract was suspended in 90% MeOH (200 ml) and partitioned with hexane (500 ml × 4). The hexane-soluble fraction was evaporated to give 15 g of a thick yellow liquid. After removal of the hexane layer, the aq. MeOH layer was concentrated to a small volume and H_2O (200 ml) was added. This mixture was extracted with EtOAc (500 ml × 4) and the EtOAc layer was evaporated to dryness to give 37 g of an orange-yellow gum. This residue was subjected to CC on silica gel (70 × 4 cm). Elution with hexane, hexane–EtOAc (9:1→1:1), $CHCl_3$, $CHCl_3$–MeOH (1:1) and finally with MeOH gave 22 fractions (200 ml each). *Z*-Guggulsterone (**4**, 300 mg) was obtained as colorless cubic crystals from Fr. # 10 by recrystallization from MeCN. Repeated column chromatography of Fr. # 11 (164 mg) over reversed phase silica gel RP-18 (eluted with MeOH–H_2O, 8:2) and subsequent preparative TLC (Si gel RP-18, system B) afforded myrrhanone A (**8**, 20 mg). Recrystallization of Fr. # 14 (34 mg) from MeCN gave fine needle crystals of guggulsterol-Y (**3**, 19 mg). Fr. # 17 (1.63 g, eluted with 30% EtOAc in hexane) was applied to a column of Si gel (eluted with increasing amount of EtOAc in hexane) to give 40 subfractions (each of 15 ml). *E*-Guggulsterone (**5**, 12 mg) was obtained from subfr. # 17 and 18 as colorless needle crystals. CC/Si gel RP-18 (eluted with MeOH–H_2O (1:1)→MeOH) of subfr. # 31–35 (349 mg) followed by recrystalization from MeCN gave guggulsterone-M (**1**, 23 mg) as fine needle crystals. While subfr. # 38 and 39 gave myrrhanol A (**7**, 100 mg). Fr. #19 (700 mg, eluted with 40% EtOAc in hexane) was applied to a column of Si gel (30 × 2 cm) using increasing amount of EtOAc in hexane to give guggulsterol-I (**6**, 150 mg) from subfr. # 38–41 as colorless needles from MeOH–H_2O (2:1). Fr. # 21 (230 mg, eluted with $CHCl_3$) was applied to a column of Si gel RP-18 and elution with MeOH–H_2O (1:1)→MeOH gave 25 subfractions. Repeated CC of subfr. # 21–23 (118 mg) on Si gel RP-18 gave dehydroguggulsterone-M (**2,** 27 mg).

3.4. Guggulsterone-M (1)

Needles (mp 206–208°, MeCN), $[\alpha]_D$ +93° (MeOH, *c* 0.65). CD (MeOH) $[\theta]_{325}+873.7\times10^3$. UV λ_{max} MeOH nm (log ε): 240 (4.3), 275 (4.5). IR ν_{max} cm^{-1}; 2938, 1668, 1635, 1590, 1450, 1378, 1226, 1078. EIMS *m/z* 342 $[M]^+$, 329 (base peak), 325, 313, 299, 285, 267, 253, 213. HR-EIMS *m/z* found 342.2191 $[M]^+$ ($C_{22}H_{30}O_3$ requires 342.2194). ^1H NMR (400 MHz, $CDCl_3$) δ: 0.98 (3H, *s*, H_3-18), 1.04 (1H, *m*, H-9), 1.12 (1H, *dd*, *J* = 4.8 and 13.1 Hz, H_b-7), 1.21 (1H, *m*, H_b-11), 1.41 (1H, *m*, H-14), 1.52 (1H, *dd*, *J* = 4.5 and 12.8 Hz, H_b-11), 1.71 (1H, *m*, H_b-1), 1.73 (1H, *m*, H-8), 1.85 (1H, *m*, H_a-7), 2.03 (1H, *m*, H_a-1), 2.05 (1H, *m*, H_b-15), 2.22 (1H, *m*, H_a-15), 2.28 (3H, *s*, H3–21), 2.30 (1H, *m*, H_b-2), 2.33 (1H, *m*, H_a-2), 2.37 (1H, *m*, H_b-2), 2.38 (1H, *dd*, *J* = 3.4 and 12.3 Hz, H_b-12), 2.42 (1H, *dd*, J = 6.7 and 12.3 Hz, H_a-12), 2.43 (1H, *m*, H_b-6), 2.53 (1H, *m*, H_a-6), 3.82 (3H, *s*, –OCH_3), 5.74 (1H, *brs*, H-4). ^{13}C NMR (see Table 2).

3.5. Dehydroguggulsterone-M (2)

Amorphous powder. $[\alpha]_D$ +36.5° (*c* 0.76, MeOH). CD (MeOH) $[\theta]_{328}+486.3\times10^3$. UV λ_{max} MeOH nm (log ϵ): 210 (3.9), 245 (3.9), 265 (4.1). IR ν_{max} cm^{-1}; 2936, 1665, 1634, 1588, 1452, 1378, 1226, 1078. EIMS *m/z* 340 $[M]^+$ (base peak), 330, 325, 313, 297, 271. HR-EIMS *m/z* found 340.2031 ($C_{22}H_{28}O_3$ requires 340.2039). ^1H NMR (400 MHz, $CDCl_3$) δ: 0.88 (3H, *s*, H_3-18), 1.02 (1H, *m*, H-9), 1.12 (1H, *m*, H_b-7), 1.14 (3H, *s*, H_3-19), 1.26 (1H, *m*, H-14), 1.51 (1H, *m*, H_b-11), 1.75 (1H, *m*, H_b-11), 1.85 (2H, *m*, H_a-7 and H_b-15), 2.12 (1H, *m*, H_a-15), 2.14 (1H, *dd*, *J* = 3.3 and 12.3 Hz, H_b-12), 2.15 (3H, s, H_3-21), 2.28 (1H, *m*, H_b-6), 2.35 ((1H, *m*, H_a-12), 2.40 (1H, *m*, H_a-6), 3.69 (3H, *s*, –OCH_3), 5.95 (1H, *d*, *J* = 1.9 Hz, H-4), 6.12 (1H, *dd*, *J* = 1.9 and 10.1 Hz, H-2), 6.94 (1H, *d*, *J* = 10.1 Hz, H-1). ^{13}C NMR (see Table 2).

3.6. Guggulsterol-Y (3)

Needles (mp 236–238 °C, MeCN). $[\alpha]_D$ −28.8° (*c* 0.5, MeOH). UV λ_{max} MeOH nm (log ε): 210 (5.1). IR ν_{max} cm^{-1}; 3334, 2946, 2476, 1666, 1461, 1375, 1058, 696. EIMS *m/z* 434 $[M]^+$, 416, 380, 333, 279, 252, 212 (base peak), 171. HR-EIMS *m/z* 434.3410 ($C_{27}H_{46}O_4$ requires 434.3424). ^1H NMR (400 MHz, $CDCl_3$–CD_3OD, 1:3) δ: 0.96 (1H, *m*, H_b-23), 0.98 (3H, *s*, H_3-26), 1.00 (3H, *s*, H_3-27), 1.05 (1H, *brd*, H-9), 1.14 (3H, *s*, H_3-19), 1.15 (1H, *m*, H-14), 1.23 (1H, *d*, *J* = 4.4 Hz, H-17), 1.24 (3H, *s*, H_3-18), 1.36 (3H, *s*, H_3-21), 1.45 (1H, *m*, H_a-23), 1.47 (1H, *m*, H_b-24), 1.51 (1H, *m*, H_b-11), 1.55 (1H, *m*, H_a-11), 1.74 (1H, *m*, H_a-22), 1.81 (1H, *dd*, *J* = 5.0 and 12.0 Hz, H_b-2), 1.83 (1H, *m*, H_b-1 and H_b-24), 1.85 (1H, *m*, H-8), 1.86 (1H, *m*, H_a-1), 1.98 (1H, *brd*, *J* = 16.2 Hz, H_b-7), 2.17 (1H, *m*, H_b-4), 2.21 (1H, *m*, H_a-7), 2.23 (1H, *m*, H_a-24), 2.24 (1H, *m*, H_a-4), 3.40 (1H, *m*, H-3), 4.56 (1H, *dt*, *J* = 4.4 and 7.5 Hz, H-16), 5.33 (1H, *brs*, H-6). ^{13}C NMR (see Table 2).

3.7. Acetylation of guggulsterol-Y (3)

To a soln. of **3** (2 mg) in anhydrous pyridine (200 μl) was added Ac_2O (200 μl) and the mixture was stirred overnight at room temperature. The mixture was then extracted with $CHCl_3$ (10 ml × 3) and the $CHCl_3$ layer was washed with H_2O (10 ml × 3) and dried over anhydrous $MgSO_4$. The $CHCl_3$ layer was evaporated to yield guggulsteronl Y diacetate (**3a**, 2.5 mg) as an amorphous powder, IR ν_{max} 3330, 1746, 1740, 1666, 1461, 1375, 1258, 1234, 1050 cm^{-1}. EIMS *m/z* 518 $[M]^+$, 459 $[M-AcOH]^+$, 417 $[M-101]^+$, 398 $[M-2AcOH]^+$, 364, 349, 297 (base peak). ^1H NMR (400 MHz, $CDCl_3$) δ: 1.96 (3H, s, –OCO–CH_3), 2.20 (3H, s, –OCO–CH_3), 4.56 (1H, m, H-3), 5.25 (1H, m, H-16), 5.29 (1H, brd, H-6).

3.8. Cell culture

The cells were maintained continuously in a 75 cm^2 plastic culture flask (Falcone, Becton Dickinson, NJ,

Table 2
^{13}C NMR spectral data of **1–3** (in $CDCl_3$)

Carbon no.	1	2	3[a]
1	35.4 *t*	155.6 *d*	36.4 *t*
2	32.6 *t*	127.5 *d*	36.0 *t*
3	199.5 *s*	186.3 *s*	72.4 *d*
4	124.0 *d*	123.9 *d*	40.9 *t*
5	170.6 *s*	168.5 *s*	140.2 *s*
6	32.6 *t*	32.6 *t*	120.2 *d*
7	31.8 *t*	30.4 *t*	30.7 *t*
8	33.3 *d*	33.2 *d*	30.2 *d*
9	54.0 *d*	51.5 *d*	49.5 *d*
10	38.7 *s*	45.0 *s*	35.6 *s*
11	20.6 *t*	22.6 *t*	19.8 *t*
12	30.4 *t*	35.1 *t*	39.5 *t*
13	44.8 *s*	43.7 *s*	41.8 *s*
14	52.0 *s*	52.6 *s*	53.9 *s*
15	38.7 *t*	33.3 *t*	30.2 *t*
16	195.5 *s*	195.4 *s*	70.3 *d*
17	125.9 *s*	125.7 *s*	58.8 *d*
18	16.0 *q*	16.0 *q*	13.3 *q*
19	17.2 *q*	18.6 *q*	17.9 *q*
20	170.7 *s*	170.5 *s*	77.2 *s*
21	31.3 *q*	31.2 *q*	24.6 *q*
22			43.3 *t*
23			21.5 *t*
24			38.8 *t*
25			76.0 *s*
26			21.1[b] *q*
27			21.2[b] *q*
20-OCH_3	57.4 *q*	57.4 *q*	

[a] Measured in 1:2 $CDCl_3$–CD_3OD. Multiplicities were verified by DEPT and HMQC experiments.
[b] Value interchangeable.

USA) in RPMI-1640 supplemented with penicillin G (100 units/ml), streptomycin (100 μg/ml) and 10% FCS. Monolayers of cells were routinely harvested by gentle scraping with a teflon cell-scraper, diluted in fresh medium and culuted to confluency at 37 °C. Confluent monolayers of J774.1 (1.5×1066 cells/well) in plastic 24-well plates (Falcone, Becton Dickinson, NJ, USA) were allowed to adhere for 4 h at 37 °C in a humidified atmosphere containing 5% CO_2. Thereafter, the medium was replaced with fresh medium containing LPS (10 μg/ml) and/or the test samples at the indicated concentrations, and the cells were incubated for 48 h under the conditions mentioned above.

3.9. Nitrite assay

Nitrite accumulation, an indicator of NO release was measured in the culture medium using a microplate assay method based on the Griess reaction (Green et al., 1982). Briefly, 400 μl of Griess reagent (1% sulfanilamide, 0.1% naphthylene-diamide dihydrochloride in 2.5% H_3PO_4) were added to equal volume of the culture medium supernatant. After 10 min of incubation at room temp., absorbance was measured at 560 nm. The nitrite concentration in the medium was calculated from a sodium nitrite standard curve freshly prepared in culture medium. Results were calculated from the mean ±s.e. of four independent determinations. The calculation of the inhibitory potency for the tested samples was done as follows:

$$\text{Inhibition}(\%) = 100 - [(Sample - Control)/(LPS - Control)] \times 100$$

where *Sample* indicates [LPS (+) and tested sample (+)], *Control* [LPS (−) and tested sample (−)], while *LPS* indicates [LPS (+) and tested sample (−)].

Curcumin was used as a positive control, which inhibited LPS-induced NO formation in J774.1 cells with an IC_{50} value of 12.3 μM under the above conditions.

Acknowledgements

The experimental work was carried out in the Institute of Natural Medicine, Toyama Medical and Pharmaceutical University, Toyama, Japan with permission of Prof. Masao Hattori and Dr. Katsuko Komatsu, for both of them the author is grateful. Thanks are also due to Dr. Javed Ahmad, Reader, Department of Botany, Jamia Hamdad (Hamdad University), New-Delhi and visiting Associate Professor of this University for authenticating the plant material, and to Mr. Sasaki Yohei for the skilled technical assistance.

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